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Neurobiology

p75 Neurotrophin Receptor-Mediated Signaling Promotes Human Hair Follicle Regression (Catagen)

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Nerve growth factor (NGF) and its apoptosis-promoting low-affinity receptor (p75NTR) regulate murine hair cycling. However, it is unknown whether human hair growth is also controlled through p75NTR, its high-affinity ligand pro-NGF, and/or the growth-promoting high-affinity NGF receptor tyrosine kinase A (TrkA). In microdissected human scalp anagen hair bulbs, mRNA for NGF, pro-NGF, p75NTR, and TrkA was transcribed. Immunohistomorphometry and *in situ* hybridization detected strong NGF and pro-NGF expression in terminally differentiating inner root sheath keratinocytes, whereas TrkA was co-expressed with p75NTR in basal and suprabasal outer root sheath keratinocytes. During spontaneous catagen development of organ-cultured human anagen hair follicles, p75NTR mRNA levels rose, and p75NTR and pro-NGF immunoreactivity increased dramatically in involuting compartments primarily devoid of TrkA expression. Here, TUNEL$^+$ apoptotic cells showed prominent p75NTR expression. Joint pro-NGF/NGF administration inhibited hair shaft elongation and accelerated catagen development in culture, which was antagonized by co-administration of p75NTR-blocking antibodies. In addition, mRNA and protein expression of transforming growth factor-β2 increased early during spontaneous catagen development, and its neutralization blocked pro-NGF/NGF-dependent hair growth inhibition. Our findings suggest that pro-NGF/NGF interacts with transforming growth factor-β2 and p75NTR to terminate anagen in human hair follicles, implying that p75NTR blockade may alleviate hair growth disorders characterized by excessive catagen development. ***(Am J Pathol 2006, 168:221–234; DOI: 10.2353/ajpath.2006.050163)***

Half a century ago, the prototypic nerve growth factor (NGF) was discovered.[1,2] Since then, almost every discipline that took up neurotrophin research unraveled new biological functions of NGF beyond the control of nerve growth.[2] NGF released systemically or locally by glands, keratinocytes, mast cells, and lymphocytes[3–5] is now established to operate as a growth factor for epithelial and mesenchymal tissues,[6–10] as an immunomodulator,[11,12] and as a key mediator of stress responses.[13,14]

The growth-promoting effect of NGF is mediated by its high-affinity tyrosine kinase receptor TrkA.[7,15,16] However, NGF signaling through TrkA can be enhanced by coupling to the low-affinity pan-neurotrophin receptor p75NTR,[17] a member of the tumor necrosis factor receptor family. In contrast, stimulation of p75NTR alone has been shown to induce apoptosis in cells lacking Trk expression,[15,17,18] on neurotrophin withdrawal,[17] or, most recently, on coupling to its newly defined high-affinity ligand, pro-NGF.[19]

The seemingly contradictory functions of NGF in the control of murine hair follicle cycling, a prototypic model of continuous tissue remodeling,[16,20,21] therefore do not surprise. In this model, p75NTR signaling has been shown to inhibit hair follicle morphogenesis and to stimulate hair follicle regression.[18,22,23] This model is characterized by the physiologically occurring cyclic remodeling of the back skin pelage hair follicles and their surrounding skin between phases of growth (anagen), regression (catagen), and relative quiescence (telogen).[20,21,24,25] In this highly instructive model, p75NTR signaling was shown to act inhibitory of hair follicle development[22] whereas NGF has been shown to promote growth of early anagen hair follicles.[26] In contrast, NGF and p75NTR signaling can induce catagen and catagen-associated apoptosis of hair follicle keratinocytes.[18,23,27] Also, NGF mediates stress-induced perifollicular inflammation and premature catagen entry in mice.[14]

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These murine data raise the expectation that NGF, via TrkA and/or p75NTR signaling, also acts as a potent hair growth modulator in humans. We have, therefore, adapted an organ culture model introduced by Philpott and colleagues[28–30] to produce and analyze catagen-like human hair follicle regression. In this model, we have studied the course of neurotrophin and neurotrophin-receptor expression through the human hair cycle with regard to the following questions:

1. Are pro-NGF and NGF able to signal within the human hair follicle?
2. What is the expression pattern of pro-NGF, NGF, TrkA, and p75NTR in human anagen VI scalp hair follicles, and how does this expression pattern change when anagen hair follicles spontaneously undergo catagen transformation in hair follicle organ culture?
3. How do these expression patterns correspond to the intrafollicular foci of keratinocyte apoptosis during catagen development in culture?
4. Can NGF promote catagen development in organ-cultured human scalp skin anagen hair follicles, while p75NTR-blocking antibodies inhibit it?
5. Do NGF effects on human anagen hair follicles involve up-regulation of the key catagen promoter, transforming growth factor-β2 (TGF-β2)?

Here, we show that NGF, pro-NGF, p75NTR, and TrkA mRNA are expressed at the transcript and protein levels in human anagen scalp skin hair follicles. The expression patterns dissected here demonstrate NGF/TrkA interactions in proliferating outer root sheath keratinocytes and pro-NGF/p75NTR interactions in the regressing catagen hair bulb corresponding to tissue foci of epithelial cell apoptosis. Pro-NGF/NGF inhibits hair shaft elongation, increases TGF-β2 transcription, and accelerates catagen development of organ-cultured human hair follicles in a p75NTR-dependent manner, with TGF-β2 likely acting downstream of NGF-induced signaling. These findings reveal an important role of the pro-NGF/NGF signaling cascade in terminating the growth of human anagen hair follicles and introduce human scalp hair follicle organ culture as an ideal, easily accessible, and highly instructive model for dissecting the complex, and often seemingly contradictory, functional effects of neurotrophin-induced signaling events in peripheral tissues.

Materials and Methods

Tissue and Hair Follicle Sources

Anagen VI hair follicles were obtained from frontotemporal scalp skin and were immediately snap-frozen in liquid nitrogen for immunohistochemistry or whole skin polymerase chain reaction (PCR) analysis or were processed for *in situ* hybridization or microdissection and hair follicle organ culture as previously described.[24,29,30,31] All samples were collected after obtaining informed consent, following accepted ethical guidelines and according to Helsinki guidelines. Of each sample, some hair follicles were immediately snap-frozen in liquid nitrogen for PCR analysis or snap-frozen embedded in a drop of OCT for histomorphometry. These follicles will be termed native hair follicles or hair follicles on day 0 throughout the remainder of the article. All other hair follicles were processed for organ culture as described below.

Conventional Reverse-Transcribed PCR Analysis

RNA was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany). Total RNA (0.8 μg) was reverse-transcribed using the First Strand cDNA synthesis kit (Roche, Mannheim, Germany) in an oligo-d$(T)_{15}$-primed 20-μl reaction. cDNA (1 μl) was amplified by PCR using the Core PCR kit (Qiagen) and the following primers: 5′-TGCATAGCGTAATGTCCATG-3′ and 5′-AAGTCCAGATCCTGAGTGTC-3′ to amplify a 325-bp fragment corresponding to nucleotides 127 to 451 of NGFB mRNA (GenBank: NM_002506), 5′-CCATCGTGAAGAGTGGTCTC-3′ and 5′-GGTGACATTGGCCAGGGTCA-3′ to amplify a 476-bp fragment corresponding to nucleotides 346 to 821 of the TrkA mRNA (GenBank: NM_001007204) as published,[32] and 5′-TGGGCAGGACCTCAGAGTCC-3′ and 5′-TTCCTCCCTCTGAGTCTCTG-3′ to amplify a 283-bp fragment corresponding to nucleotides 1517 to 1799 of p75NTR mRNA (GenBank: NM_002507). The reactions were cycled for 5 minutes at 95°C, then 30 seconds at 95°C, 30 seconds at 60°C, 45 seconds at 72°C for 35 cycles, and finally 5 minutes at 72°C.

Quantitative Real-Time PCR Analysis

For analysis of TGF-β2 and p75NTR mRNA during catagen-like hair follicle regression in cultured human anagen hair follicles, hair follicles were snap-frozen immediately after isolation (day 0) or after 4, 8, or 12 days in culture with complete Williams E medium (Table 1). Hair follicles cultured throughout 48 hours in the presence of 50 ng/ml of 7S NGF from mouse submaxillary gland (Sigma, St. Louis, MO) (Table 1) were also snap-frozen and processed as described above.

The assay for the amplification of tumor growth factor β2 (TGF-β2) and p75NTR exploits the 5′nuclease activity of AmpliTaq Platin (Invitrogen, Karlsruhe, Germany) DNA polymerase to cleave a fluorogenic probe designed for pro-NGF/NGF (TipMolBiol, Berlin, Germany) and, to normalize our samples, a fluorogenic probe for the housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT) was used in real-time PCR. The sets of primers and probes were designed as follows: TGF-β2 probe: sequence 5′-6FAM-ACACGAACCCAAAGGGTACAATGCCAXT–PH-3′, reverse primer 5′-ATTGATTTCAAGAGGGATCTAGGGTG-3′, forward primer 5′-CAGGACCCTGCTGTGCTG-3′; p75NTR probe: sequence 5′-6FAM-CTCGGGCCTCGTGTTCTCCTGCXT–PH-3′, reverse primer 5′-TGGCCTCGTCGGAATACG-3′, forward primer 5′-CTTACGGCTACTACCAGGATGAG-3′; HPRT probe: sequence 5′-6FAM-CAAGCTTGCTGGTGAAAAGGACCCCXT–PH-3′, reverse primer 5′-GTCTGGCTTATATCCAACACTTCGT-3′, forward primer 5′-

Table 1. Hair Follicle Organ-Culture Treatment Schedules

Peptide	Specification	Source	Treatment	No. of scalp skin specimens from different donors
None[29]	Complete Williams E hair follicle growth medium as published by Philpott and colleagues[29]	See Materials and Methods	Medium change every 2nd day. Harvested after 0, 4, 8, or 12 days for PCR and IHC	12
NGF 7S[32]	Isolated from mouse submaxillary gland, active in human	Sigma, St. Louis, MO	5 or 50 ng/ml every other day. Harvested day 2, 4, or 10 for PCR (only day 4) and IHC	10 (day 2, $n = 1$; day 4, $n = 2$; day 10, $n = 7$)
p75NTR-neutralizing antibody[50]	Monoclonal mouse IgG from ME20.4 hybridoma against human melanoma cell line WM245. recognizes an epitope within the third or fourth cysteine-rich repeats of p75NTR	Sigma	5 or 50 ng/ml every other day. Harvested day 10 for IHC	8 (medium control growth = 100%, $n = 4$; medium control growth = 60%, $n = 4$)

The table gives information on growth factors and neutralizing antibodies used in organ-cultured human scalp skin anagen hair follicles. It also informs on timing of medium change, termination of culture (harvesting), and number of samples derived from different donors. Each donor donated nine hair follicles per experiment. IHC, immunohistochemistry.

GGCAGTATAATCCAAAGATGGTCAA-3′. The real-time PCR reactions were developed as previously published[33] except that we used 0.2-μl probe at 20 μmol/L. Each analysis was normalized to HPRT by calculating the difference between the CT for HPRT and the CT for p75NTR or TGF-β2 as ΔCT = CT HPRT − CT p75NTR or TGF-β2. Amount of mRNA is expressed as increase or decrease over native hair follicles = 1.

For the amplification of TGF-β2 from pro-NGF/NGF-cultured hair follicles, reactions were performed as 50-ml triplicates in a 96-well microplate format containing 1× SYBR Green Master Mix (PE Biosystems, Foster City, CA), first strand cDNA, and each primer set. Primers were designed using the Perkin Express 1.0 Software (PE Biosystems) and each set of primers are situated in different exons as human TGF-β2 5′-AAAGTGGACG-TAGGC AGCAATTA-3′ forward primer, human TGF-β2 5′-GACCAACCGGCGGAAGA-3′ reverse primer, human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 5′-TGGGTGTGAACCATGAGAAG-3′ forward primer, and human GAPDH 5′-GCTAAGCAGTTGGTGGTGC-3′ reverse primer, respectively. The reaction mixture was subjected to the following thermal cycle conditions: 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Analysis of the reactions was performed in an ABI Prism 7700 sequence detection system (PE Biosystems) monitoring after each cycle. The expression of TGF-β2 was normalized with that of GAPDH. Each TaqMan experiment was performed in triplicate.

In Situ *Hybridization*

Oligonucleotide probes to p75NTR, TrkA, and pro-NGF/NGF, labeled with fluorescein at both the 3′ and 5′ ends, were designed by and obtained from Biognostik (Göttingen, Germany). Paraffin sections (4 μm thick) were pretreated with proteinase K (Roche) and postfixed with 1% paraformaldehyde in phosphate-buffered saline (pH 7.4). Sections were then prehybridized in hybridization buffer [2× standard saline citrate (0.15 mol/L NaCl, 0.015 mol/L sodium citrate, pH 7.0), 500 ng/ml yeast tRNA (Sigma, Taufkirchen, Germany), 150 mmol/L NaCl, 50% formamide]. Hybridization was performed with 1.5-pmol probe in the hybridization buffer. Probes were detected using anti-fluorescein AP FAB fragments (Roche) in a standard NBT/BCIP color reaction.

Immunohistochemistry

Cryostat sections (8 μm) fixed in acetone (at −20°C, 10 minutes), were preincubated with 10% normal goat serum and then incubated with the primary antisera to pro-NGF, NGF, TrkA, and p75NTR (Table 2) as described.[31] Tetramethyl-rhodamine-isothiocyanate-conjugated $F(ab)_2$ fragments of goat anti-rabbit IgG (Dianova, Hamburg, Germany) served as secondary antibodies. Then sections were stained with 4,6-diamidino-2-phenylindole (DAPI; Boehringer Mannheim, Mannheim, Germany) for identification of cell nuclei.

Two types of negative controls were run. Slides were incubated with the secondary antibody alone or with a mixture of the primary antibody and the control peptide for the specific antisera [incubated overnight at 4°C in tubes covered with 1% milk powder in Tris-buffered saline (TBS)] (20 times more concentrated than primary antibody, overnight, room temperature; Santa Cruz Biotechnology, Santa Cruz, CA) after preincubation with 10% normal goat serum. Labeling of nerve fibers and nerve fiber bundles (pro-NGF, NGF, p75NTR) and basal epidermal keratinocytes (TrkA) in full-thickness skin biopsies served as intrinsic positive controls.

Table 2. Antibodies for Immunofluorescence

Antigen	Specification	Source	Dilution/protocol	No. of examined individuals
Pro-NGF	Rabbit, polyclonal, raised against murine pro-NGF	Chemicon, Temecula, CA	1:150 Immunofluorescence	8
NGF	Rabbit, polyclonal, raised against a peptide mapping to hNGF N-terminus	Santa Cruz Biotechnology, Santa Cruz, CA	1:50 Immunofluorescence	12
NGF	Goat, polyclonal, raised against rhβ-NGF	R&D Systems, Minneapolis, MN	1:20 Immunofluorescence	8
NGF	Mouse, monoclonal, raised against rhβ-NGF	Sigma, St. Louis, MO	In our hands no satisfactory immunostaining results	3
NGF	Rat, polyclonal, raised against hNGF	Promega, Madison, WI	In our hands no satisfactory immunostaining results	4
TrkA	Rabbit, polyclonal, raised against AA 763-777 within the C-terminus of Trk gp 140	Santa Cruz Biotechnology	1:75 Immunofluorescence	12
p75NTR	Mouse, monoclonal, raised against human melanoma cell line WM245, reacts against the third or fourth cysteine-rich repeats	Sigma	1:400 Immunofluorescence	4
p75NTR	Mouse, monoclonal (production discontinued)	Chemicon	1:500 Immunofluorescence	12
p75NTR	Rabbit, polyclonal	Chemicon	1:1000 TSA	7

This table lists all antibodies employed for immunohistochemical detection of NGF, pro-NGF, TrkA, and p75NTR. For NGF we found two working antibodies yielding comparable staining results that mapped different epitopes of NGF. For pro-NGF only one antibody has recently been brought on the commercial market. To our knowledge, this is the first report on pro-NGF in human skin with this antibody. For TrkA only antibodies mapping the same epitope were commercially available. Both antibodies were available from Santa Cruz Biotechnology, we therefore chose these for immunofluorescence screening of all available skin samples. Three different antibodies yielding highly comparable results confirmed p75NTR immunoreactivity patterns.

Histomorphometry

Stained sections were examined at ×250 magnification under a Zeiss Axioscope 2 microscope with a fluorescence device (Zeiss, Göttingen, Germany). *In situ* labeling or immunoreactivity (IR) was quantified by histomorphometry in the following compartments: the hair follicle ostium (hair follicle epithelium between epidermis and entry of the sebaceous gland into the hair canal), inner root sheath, outer root sheath, cuticle, dermal papilla, matrix, proximal hair bulb epithelium (below Auber's line), sebaceous gland, epidermis (separated into stratum basale, spinale, and granulosum), arrector pili muscle, sweat gland, blood vessels, nerve fibers, and melanocytes. Staining intensities were documented in arbitrary units as follows: 0 = negative, + = barely visible staining, ++ = apparent staining, +++ = strong staining. For each IR pattern follicular compartments were evaluated in at least four different anagen hair follicles per sample and the extrafollicular compartments in a minimum of 10 microscopic fields. The photodocumentation was done with the help of a modular imaging program (Openlab; Improvision, Heidelberg, Germany). The results of the observed, representative IR patterns were also recorded qualitatively in schematized recording protocols.

Hair Follicle Organ Culture

Three microdissected human scalp skin anagen hair follicles per donor and experiment were randomly placed per well and cultured in 24-well plates (Costar, Corning, NY) containing 500 ml of complete hair follicle culture medium per well. Per experiment a minimum of three wells (containing three hair follicles each) was assigned to each test group and was supplemented with different concentrations of pro-NGF/NGF, p75NTR-neutralizing antibody, or TGF-β-neutralizing antibody or pro-NGF/NGF in combination with either anti-p75NTR or anti-TGF-β (Table 1). Please note, that all commercially available NGF preparations tested to date contain pro-NGF along with NGF whether they are derived from mouse submaxillary gland or are recombinant.[34] The 7S NGF used in our study will therefore be referred to as pro-NGF/NGF throughout the article. The p75NTR-neutralizing antibody was used to neutralize p75NTR-mediated pro-NGF/NGF signaling because we had no access to specific TrkA agonists or antagonists or to the p75NTR antagonist we had used in earlier studies.[24] To further dissect NGF-signaling pathways in hair growth control, we also used culture of hair follicles in the presence of 5 or 50 ng/ml pro-NGF/NGF and TGF-β-neutralizing antibody (monoclonal anti-TGF-β 1-3 antibody, binds to all three TGF-β isoforms; R&D Systems, Minneapolis, MN).[31]

Every second day, each well was photodocumented, the total length of each hair follicle measured, medium replaced, and fresh supplements added. After 2, 4, 8, or 10 days, hair follicles were snap-frozen in a drop of OCT Cryochrome (Shandon, Pittsburgh, PA) and stored at −80°C until cryosectioning. Some additional control samples (Table 1) were kept in culture for 12 days to observe spontaneous catagen-like regression,

which regularly occurs in this model between the day 8 and 12 in culture.

Hair Cycle Staging

Cultured hair follicles were staged after 10 days (Table 2) in culture by routine hematoxylin and eosin (H&E) histomorphometry, following previously published morphological characteristics of the murine and human hair cycle.[24,31,35] Briefly, hair follicles were classified as anagen VI, early catagen, mid-catagen, or late catagen. For statistical analysis (hair cycle score), anagen VI hair follicles were arbitrarily attributed a score of 100, hair follicles in early catagen a score of 200, in mid-catagen of 300, and in late catagen of 400. The sum of scores per group was then divided by the number of investigated hair follicles.[36,37] The mean value of these scores therefore is a reliable quantitative indicator of the mean hair follicle stage that had been reached on average by a larger population of hair follicles after culture.[36,37]

Apoptosis/Proliferation Assay

Cryostat sections of cultured hair follicles were processed for terminal dUTP nick-end labeling (TUNEL) (apoptotic cell nuclei, Apopdetect Fluorescein; QBiogene, Heidelberg, Germany) and Ki67-labeling (proliferating cell nuclei, monoclonal mouse anti-human Ki-67 antigen; DAKO, Hamburg, Germany) after 2, 4, or 10 days (Table 2) according to our previously published protocol,[27] adapted for human antigens. Hair bulbs were again photodocumented and histomorphometry was performed as described above on all hair follicles, allowing full appreciation of a longitudinal-sectioned dermal papilla (since this is very difficult to achieve, the total number of hair bulbs analyzed was restricted to 8 to 14 hair bulbs per group).

Statistical Analysis

Hair follicle elongation, hair cycle scores, TUNEL IR, and Ki67 IR cell nuclei numbers were pooled per group for each experiment. The results between different experiments using samples from different donors were highly comparable so that the mean scores and numbers of TUNEL IR or Ki67 IR cell nuclei per group and experiment from all experiments were then pooled again and statistical differences between groups were determined by Mann-Whitney *U*-test for unpaired samples.

Results

Human Anagen Hair Follicles Express Pro-NGF/NGF, TrkA, and p75NTR mRNA

Reverse-transcriptase (RT)-PCR revealed that microdissected human scalp skin anagen hair follicles, which included the hair follicle epithelium proximal to the isthmus and bulge region as well as the dermal papilla and the proximal connective tissue sheath of the hair follicle, contained transcripts of the pro-NGF/NGF gene, encoding both pro-NGF and mature β-NGF. We were also able to detect an mRNA signal both for TrkA and p75NTR

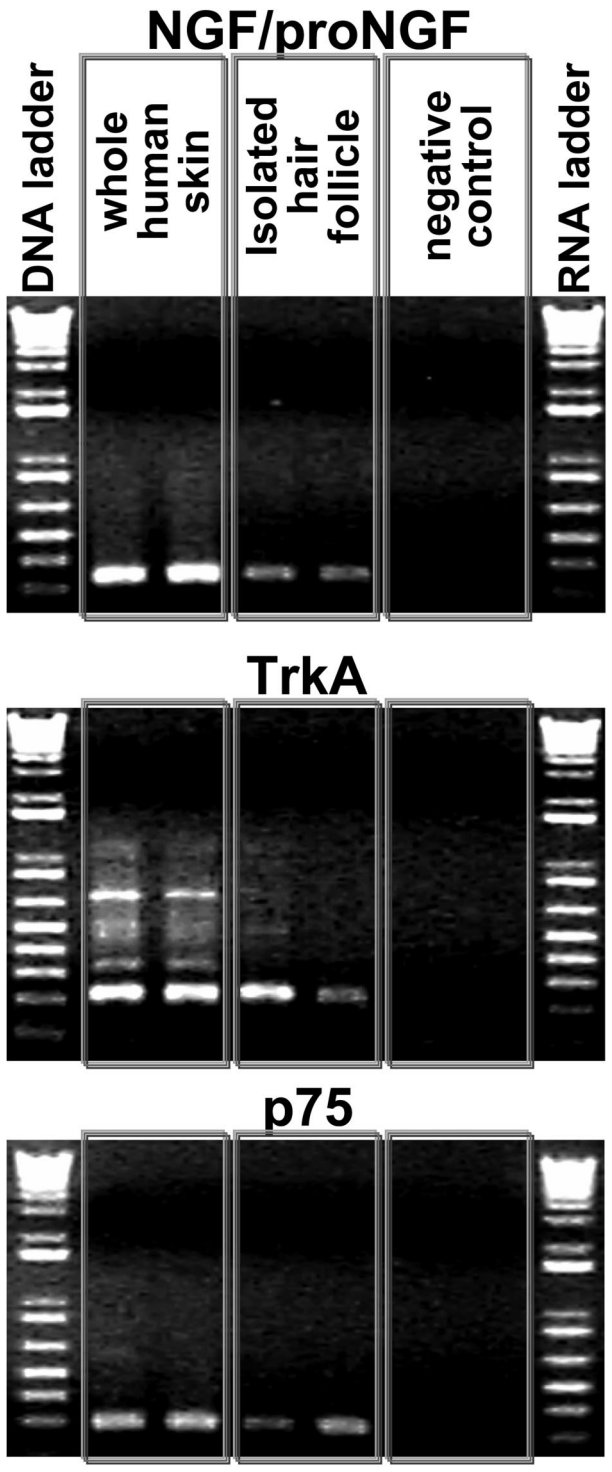
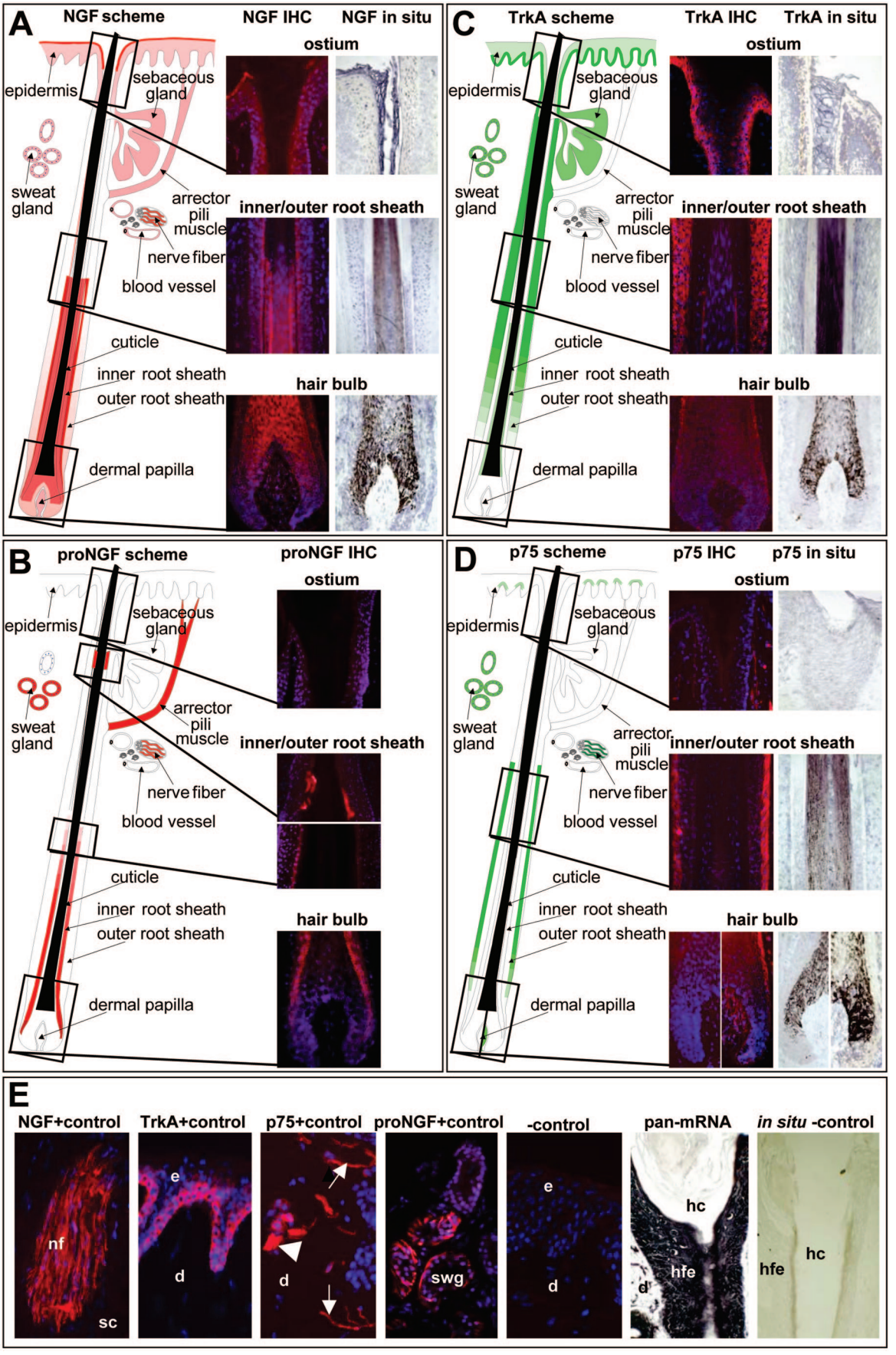


Figure 1. Pro-NGF/NGF, TrkA, and p75NTR are expressed in the human anagen hair follicle by reverse-transcribed PCR. Reverse-transcribed PCR analysis was performed on isolated human hair follicles using primers for pro-NGF/NGF, TrkA, and p75NTR. As positive control RNA from full-thickness temporal human scalp skin was used and water was used as negative controls.


A
NGF scheme
NGF IHC
NGF in situ
ostium
epidermis
sebaceous gland
sweat gland
arrector pili muscle
inner/outer root sheath
nerve fiber
blood vessel
cuticle
inner root sheath
outer root sheath
hair bulb
dermal papilla
C
TrkA scheme
TrkA IHC
TrkA in situ
B
proNGF scheme
proNGF IHC
D
p75 scheme
p75 IHC
p75 in situ
E
NGF+control
TrkA+control
p75+control
proNGF+control
-control
pan-mRNA
in situ -control
nf
sc
e
d
swg
hc
hfe

(Figure 1). Thus, the human anagen scalp skin hair follicle holds the full capacity for autocrine and paracrine pro-NGF/NGF-signaling.

NGFs and Their Receptors Are Differentially Expressed within Defined Hair Follicle Compartments

Immunohistochemistry and *in situ* hybridization revealed strongest expression of NGF and its precursor in terminally differentiating inner root sheath keratinocytes of human anagen scalp skin hair follicles in full-thickness skin samples (Figure 2, A and B; Table 3). NGF expression was also found in the most distal layer of the epidermis and in the matrix of the hair bulb whereas strong pro-NGF IR was found in the terminally differentiated distal inner root sheath (Figure 2, A and B; Table 3). In the anagen hair follicle, weak NGF expression, but no pro-NGF, was detected in the dermal papilla. Outside the hair follicle epithelium, both markers were found in nerve fiber bundles as expected, and pro-NGF expression was also strong in smooth muscle cells.

TrkA as well as p75NTR were expressed in the basal and suprabasal layers of the proximal outer root sheath of human anagen scalp skin hair follicles in full thickness skin samples, whereas TrkA expression dominated in the distal outer root sheath (Figure 2, C and D; Table 3). Neither was found in the hair follicle matrix nor in the dermal papilla of late anagen hair follicles, as assessed by immunohistochemistry and *in situ* hybridization. However, few hair follicles were found to fulfill the criteria of early catagen hair follicles, and these hair follicles displayed faint p75NTR expression in the hair follicle matrix and strong expression in the dermal papilla (Figure 2D, Table 3). Positive and negative controls showed the expected reactivity patterns (Figure 2D).

Catagen-Like Hair Follicle Regression in Culture Is Characterized by p75NTR/TUNEL Co-Expression in the Regressing Hair Bulb

Human anagen scalp skin hair follicles were left to grow in complete hair follicle culture medium for 12 days without additional treatments. This culture period was long enough for human anagen hair follicles to spontaneously enter a catagen-like regression phase.[28–30,38–40] This catagen-like regression was identified by the classical morphological features of catagen development *in situ*[24,35,41] and lacked signs of necrosis as evidenced by TUNEL labeling (Figure 3). Briefly, we observed rounding up of the dermal papilla, progressive loss of keratinocytes from the hair bulb resulting in substantial reduction of hair bulb diameter and keratinocyte-to-dermal papilla fibroblast ratio, termination of hair shaft production, and formation of a club-like ending of the hair shaft surrounded by an epithelial sack and connected to the dermal papilla by an epithelial strand. By these criteria, hair follicles could be subclassified into early, mid-, and late catagen-like hair follicles (Figure 3)[35,41] and are termed accordingly throughout the remainder of the article.

During this catagen-like hair follicle involution in organ culture, we observed a dramatic increase in pro-NGF expression in the inner root sheath, the hair follicle matrix and, during the late catagen-like stages, in the developing epithelial sack and club hair (Figure 3). Low pro-NGF-expression was now also present in the dermal papilla where we could not detect it in anagen VI hair follicles, neither *in situ* (compare Figure 2B) nor after culture (not shown). Interestingly, hair matrix keratinocytes directly adjacent to the dermal papilla and in the most proximal hair bulb remained pro-NGF-negative (Figure 3). In the pro-NGF$^+$ compartments, TUNEL$^+$ cells became increasingly detectable in mid- and late catagen-like hair follicles (Figure 3), and these cells strongly expressed the p75NTR (Figure 3), which also lit up the entire epithelial strand and club hair follicle of late catagen-like hair follicles.

All compartments that displayed strong pro-NGF and p75NTR expression showed weak or no TrkA expression, especially in the hair follicle matrix of mid-catagen-like hair follicles and in the epithelial strand of late catagen-like hair follicles (Figure 3). TrkA expression remained restricted to the regressing outer root sheath of early and mid-catagen-like hair follicles. In mid-catagen-like hair follicles, this expression extended to form a continuous, single TrkA$^+$ hair follicle epithelial cell layer residing directly adjacent to the basement membrane that separates the hair bulb epithelium from the dermal papilla and the connective tissue sheath. This compartment was the last to harbor proliferating Ki67$^+$ cells and corresponded with the pro-NGF-negative compartment in mid-catagen-like hair follicles (Figure 3). The late catagen-like hair follicles were devoid of TrkA expression with the exception of single cells in the epithelial strand, as previously described in the mouse.

Figure 2. NGF, TrkA, pro-NGF, and p75NTR expression patterns in human hair follicles. The schematic summaries display the most highly reproducible NGF-, pro-NGF-, TrkA-, and p75NTR-IR patterns revealed in human anagen scalp skin hair follicles. Cryosections (IHC) or paraffin sections (*in situ* hybridization) from human scalp skin samples containing 80 to 90% anagen hair follicles were stained with antisera against NGF, pro-NGF, TrkA, or p75NTR (Table 2) or were labeled with oligonucleotide probes to pro-NGF/NGF, TrkA, or p75NTR (for details see Materials and Methods). All images demonstrate representative IR patterns in anagen hair follicles. **Boxes** indicate anatomical location of the presented micrographs. Colors in schematic drawings and bright red fluorescence in photomicrographs indicate localization of positive IR. The **bottom panel** gives examples of positive and negative controls. For the IHC-negative control, the primary antibody was omitted and the secondary antibody was applied (identical for all stainings). For NGF, a subcutaneous nerve fiber bundle served as positive control. For TrkA the strongly positive basal layer of the epidermis served as positive control. For p75NTR single nerve fibers (**arrow**) and small nerve fiber bundles (**arrowhead**) in the subepidermal dermis served as positive control. For pro-NGF sweat glands served as positive control. Pan-mRNA served as positive and omittance of probe as negative controls for *in situ*. d, dermis; e, epidermis; hc, hair canal; hfe, hair follicle epithelium; nf, nerve fiber; sc, subcutis; swg, sweat gland.

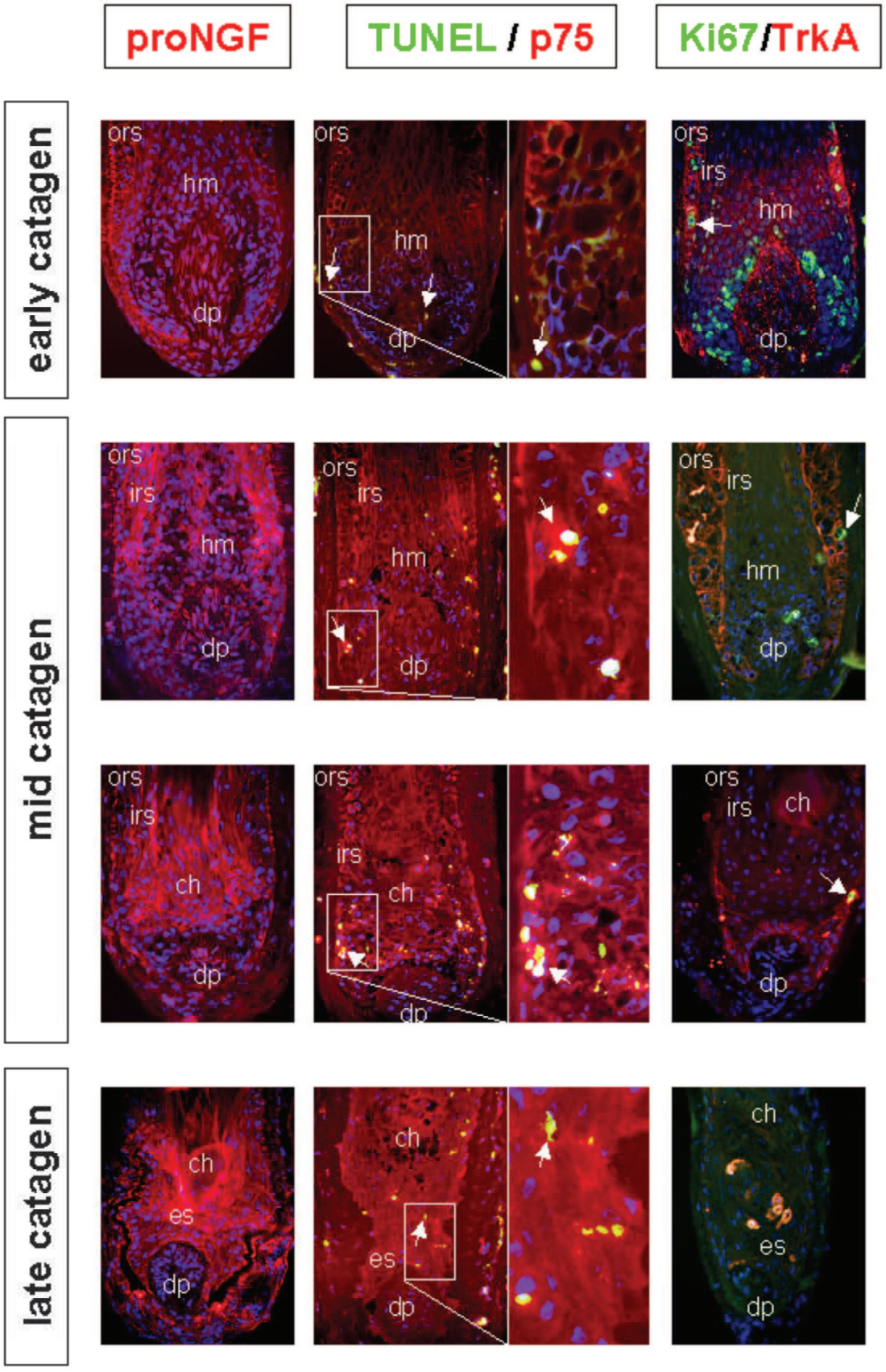

proNGF
TUNEL / p75
Ki67/TrkA
early catagen
mid catagen
late catagen
ors
irs
hm
dp
ch
es

Table 3. Summary of NGF, pro-NGF, TrkA, and p75NTR Staining Intensities in Various Compartments of Anagen VI Scalp Skin Hair Follicles

	pro-NGF	NGF	TrkA	p75NTR
Hair follicle	−	+	+++*	−
Hair follicle-ostium				
Inner root sheath	+++	++	0	−
Proximal outer root sheath	−	+ to ++†	+++	+++
Cuticle	−	++	+	−
Dermal papilla	−	+	0	−/++‡
Matrix	−	0	0	−/+¶
Proximal hair follicle bulb	−	++§	0	−
Sebaceous gland	−	+	++	−
Extrafollicular				
Epidermis stratum corneum	−	+++	0	−
Epidermis, stratum spinale	−	+	+	−
Epidermis, stratum basale	−	+	+++	+++¶
Arrector pili muscle	+++	+	0	−
Sweat gland	+++‖	+	++	+
Blood vessel	−	+	0	−
Nerve fibers	++	++ to +++	0	+++
Melanocytes	−	0	++	−

The table lists the mean staining intensities from 8 (pro-NGF) or 12 (NGF, TrkA, p75NTR) different human scalp skin samples. From each sample 10 microscopic fields or 6 hair follicles were evaluated and assigned arbitrary scores (+, weak; ++, obvious staining; +++, strong staining).

*Basal layer, rest is +-like epidermis.

†Innermost layer, rest is −.

‡In some very early catagen-like hair follicles.

§With the exception of the cell layer adjacent to the dermal papilla, which contains the hair follicle melanocytes.

¶Only on the tips of the papillae.

‖Accompanying smooth muscle cells.

Catagen-Like Hair Follicle Regression in Culture Is Characterized by Temporally Controlled p75NTR mRNA Up-Regulation

mRNA was extracted from human anagen scalp skin hair follicles after organ culture in complete hair follicle culture medium for 0 (native, anagen VI), 4 (early catagen-like development), 8 (mid-catagen-like development), or 12 (late catagen-like development) days without additional treatments. In this *in vitro* assay for spontaneous catagen development, p75NTR mRNA increased dramatically in the late phase of catagen-like regression (Figure 4), during which we observed p75NTR co-expression with TUNEL.

High Levels of Pro-NGF/NGF Inhibit Hair Shaft Elongation and Promote Catagen-Like Regression in Cultured Human Scalp Skin Anagen Hair Follicles

7S NGF (50 ng/ml), which contains pro-NGF along with NGF, inhibited hair shaft elongation in organ-cultured human anagen scalp skin hair follicles (Figure 5, A and B). By contrast, 5 ng/ml of 7S NGF did not inhibit hair shaft elongation, and a mild but not significant increase in hair shaft elongation could be observed (Figure 5, A and B). In addition, 50 ng/ml of 7S NGF, but not 5 ng/ml, induced premature entry into a catagen-like hair cycle stage in cultured human scalp skin anagen hair follicles as evidenced by determination of the hair cycle score (Figure 5C).

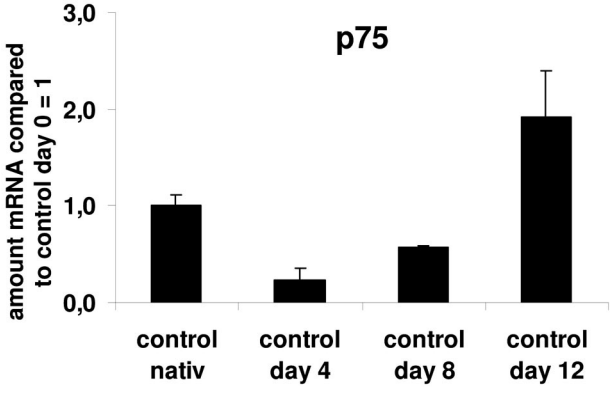


Figure 4. p75NTR mRNA levels in cultured human hair follicles in anagen-catagen-like transition. TaqMan reverse-transcribed PCR data are displayed from hair follicles derived from the same donors harvested on day 0 (native, anagen VI, $n = 3 \times 3$ hair follicles) or after 4 (early catagen-like, $n = 5 \times 3$ hair follicles), 8 (mid-catagen-like, $n = 1 \times 3$), or 12 (late catagen-like, $n = 2 \times 3$) days in culture, respectively. Each extraction contained three hair follicles, was measured in duplicate, and produced highly reproductive results. Amount of mRNA is presented in arbitrary units compared to control levels, when control levels equal 1.

Figure 3. NGF-, TrkA-, pro-NGF, and p75NTR expression patterns in cultured human catagen-like hair follicles. The photomicrographs display the most highly reproducible pro-NGF, p75NTR/TUNEL, and TrkA/Ki67 IR patterns revealed in human anagen scalp skin hair follicles undergoing spontaneous catagen-like regression in culture. Cryosections from cultured human anagen hair follicles were stained with antisera against pro-NGF (red), p75NTR (red) in double staining with TUNEL (green), or TrkA (red) in double staining with Ki67 (green). Cell nuclei have been counterstained blue with DAPI. All images demonstrate representative IR patterns in catagen-like hair follicles. If not otherwise labeled, **arrows** point at double-labeled cells. **Boxes** indicate anatomical location of selected magnifications. ch, club hair; dp, dermal papilla; e, epidermis; es, epithelial strand; hm, hair matrix; irs, inner root sheath; ors, outer root sheath.

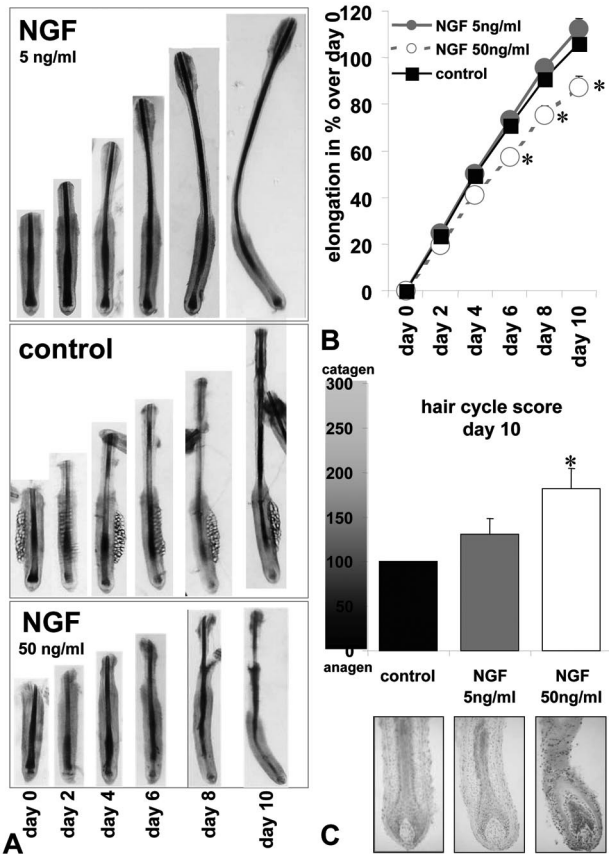


Figure 5. Pro-NGF/NGF modulates hair shaft elongation in organ culture. Per group three wells each containing three hair follicles were cultured per donor. **A:** Photodocumentation panels throughout a culture period of 10 days of one characteristic hair follicle per group are given. **B:** Data pooled from seven highly comparable, independent experiments (different donors) is shown. Growth rates are expressed as percent difference over day 0. Significances always refer to the growth rate of control hair follicles on the same day (*P* values by Mann-Whitney *U*-test: $<0.05 = +$). **C:** Characteristic examples of photodocumented hair follicles cultured throughout 10 days and corresponding H&E-stained cryosections. Note the wide anagen hair bulb of the control hair follicle (**left**). In comparison, the hair bulb of a hair follicle treated with 50 ng/ml rhBDNF appears narrow, the dermal papilla has rounded up and the hair follicle epithelium is not enclosing it. The graph represents data pooled from four highly comparable independent experiments (nine hair follicles per experiment). Data are represented as percentage hair follicles in anagen VI, early or late catagen, respectively, or as hair cycle score. Each anagen VI hair follicle was ascribed an arbitrary value of 100, early catagen-like of 200, mid-catagen-like of 300, and late catagen-like of 400 to calculate the hair cycle score. Values were added per group and divided by the number of staged follicles. The score thus represents the mean hair cycle stage of all hair follicles per group. Significances refer to the hair cycle score of control hair follicles on the same day (*P* values by Mann-Whitney *U*-test: $<0.001 = +++$, $<0.01 = ++$, $<0.05 = +$). c, club; dp, dermal papilla; hs, hair shaft; irs, inner root sheath; ors, outer root sheath; pu, pigmentary unit.

Pro-NGF/NGF Inhibits Proliferation and Promotes Apoptosis in Keratinocytes of Cultured Human Scalp Skin Hair Follicles

Hair follicles cultured in the presence of 5 or 50 ng/ml of 7S NGF showed significantly more Ki75$^+$ proliferating cells in the hair bulb after 2 days in culture (Figure 6). However, with extension of the culture period, the number of proliferating cells was reduced to levels not differing from control in hair follicles treated with 50 ng/ml of 7S NGF after 4 days in culture and in hair follicles treated

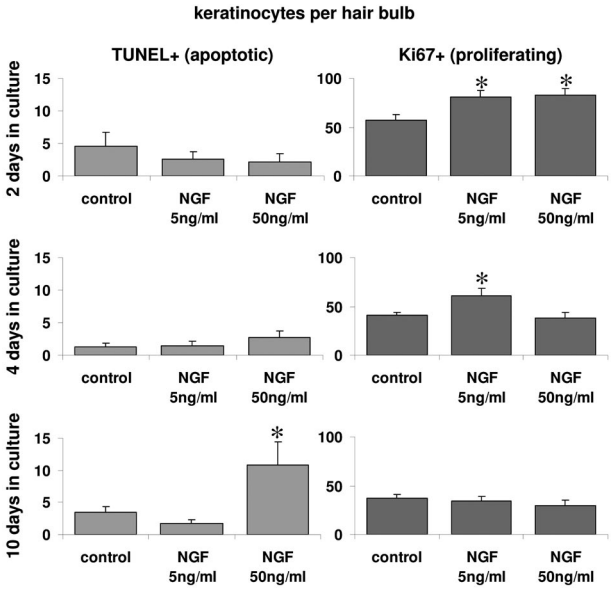


Figure 6. TUNEL/Ki67 double-staining analysis on cultured hair follicles reveals decreased proliferation and increased apoptosis after 10 days in culture with 50 ng/ml 7S NGF. Per experiment harvested one to four of nine hair follicles allowed quantification of TUNEL-IR (light) or Ki67-IR (dark) nuclei. Data from days 2 and 4 represent one donor each, data from day 10 after onset of culture was pooled from four donors. Only analysis of apoptosis and proliferation in keratinocytes yielded significant differences, data from dermal papilla and connective tissue sheath fibroblasts is therefore not shown. Significances always refer to control levels (*P* values by Mann-Whitney *U*-test: $<0.05 = +$).

with 5 ng/ml of 7S NGF after 10 days in culture (Figure 6). By contrast, TUNEL$^+$ apoptotic cells in the hair bulb were relatively low after 2 and 4 days in culture but increased dramatically in hair follicles cultured in the presence of 50 ng/ml of 7S NGF (Figure 6).

Pro-NGF/NGF Effects on Cultured Human Scalp Skin Hair Follicles Can Be Neutralized by p75NTR-Neutralizing Antibody Treatment

When hair follicles were cultured in the presence of 50 ng/ml of 7S NGF, addition of the p75NTR-neutralizing antibody at a concentration of 5 ng/ml completely reversed the growth inhibitory effect of pro-NGF/NGF (Figure 7A). Moreover, on day 4 in culture, growth of hair follicles cultured with 50 ng/ml of 7S NGF and 5 ng/ml p75NTR-neutralizing antibody was significantly better than growth of controls (Figure 7A). However, hair follicles cultured in the presence of 50 ng/ml of 7S NGF and 50 ng/ml of p75NTR-neutralizing antibody showed hair shaft elongation rates significantly below controls (Figure 7A), as did hair follicles cultured in the presence of 50 ng/ml of 7S NGF alone (compare Figure 5).

Interestingly, when hair follicles with good control growth rates (mean, ~100%; used for standard hair follicle organ culture) were cultured in the presence of p75NTR-neutralizing antibody alone, hair follicles showed reduced hair shaft elongation (Figure 7B). By contrast, hair follicles with low control growth rates (mean, ~60%; excluded from standard hair follicle analysis) showed improved hair shaft elongation after treat-

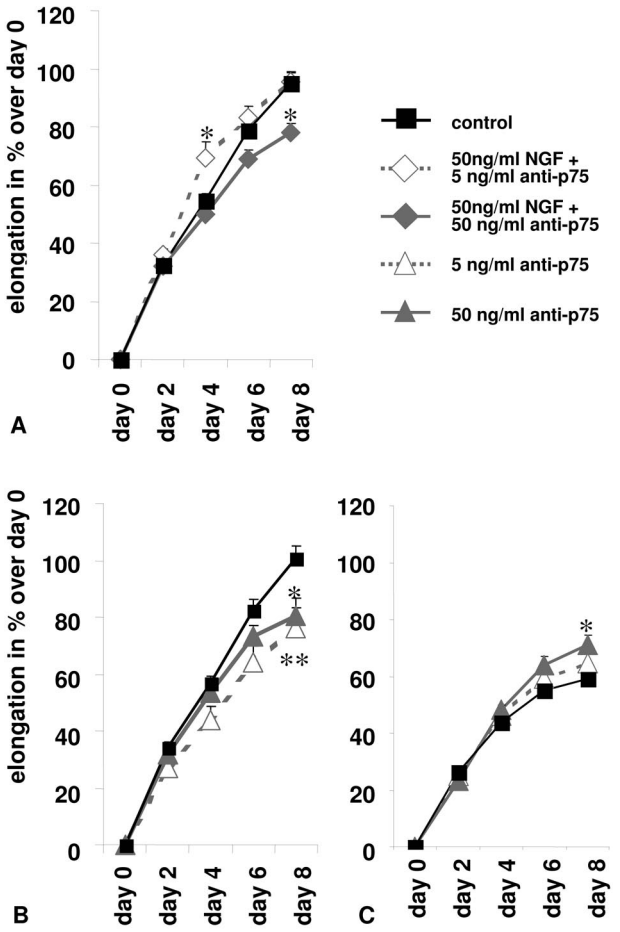


Figure 7. P75NTR-neutralizing antibody abrogates pro-NGF/NGF-induced inhibition of hair shaft elongation and promotes hair shaft elongation in submaximally growing cultures. Data pooled from one (**A**) or four (**B** and **C**) independent donors (nine hair follicles per experiment) is shown. Growth rates are expressed as percent difference over day 0. Significant differences always refer to the growth rate of control hair follicles on the same day. Note, that **C** represents hair follicle cultures normally excluded from analysis because of the low growth rate in untreated control hair follicles (mean only ~60%). This low control growth rate indicates ill-treatment of the scalp skin sample during transport and hair follicle isolation (long transport period, lack of cooling, long isolation period, suboptimal pH of medium during transport, and isolation) so that hair follicles are primed to enter into a catagen-like stage and cease growing before culture and do so earlier than under optimal conditions.

ment with 50 ng/ml of p75NTR-neutralizing antibody (Figure 7C).

The Catagen-Promoter TGF-β2 Acts Downstream of Pro-NGF/NGF-Induced Catagen Induction

As a marker for catagen induction,[31,32,42] increased TGF-β2 expression was found in the hair follicle matrix and dermal papilla of mid-catagen-like hair follicles (Figure 8A) similar to the pro-NGF-expression (compare Figure 3). In addition, the pro-NGF-negative proximal hair bulb of mid-catagen-like hair follicles was also TGF-β2-positive. By contrast, the late catagen-like hair follicle showed only weak TGF-β2 reactivity (Figure 8A). This IR pattern corresponded to the TGF-β2 mRNA expression

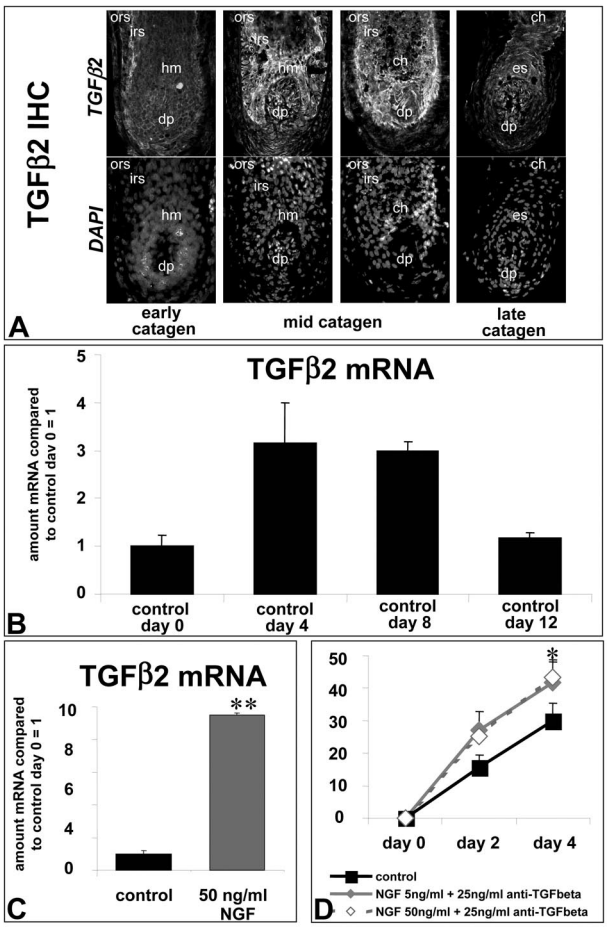


Figure 8. TGF-β2 is increased in spontaneous catagen-like development and after pro-NGF/NGF treatment and its neutralization inhibits pro-NGF/NGF-induced catagen-like development. **A:** The photomicrographs display the most highly reproducible TGF-β2 IR patterns revealed in human anagen scalp skin hair follicles undergoing spontaneous catagen-like regression in culture. Cryosections from cultured human anagen hair follicles were stained with antiserum against TGF-β2 (**top row**). Cell nuclei have been counterstained with DAPI (**bottom row**). All images demonstrate representative IR patterns in catagen-like hair follicles. **B:** TaqMan PCR data are displayed from hair follicles derived from the same donors harvested at day 0 (native, anagen VI, $n = 3 \times 3$ hair follicles) or after 4 (early catagen-like, $n = 5 \times 3$ hair follicles), 8 (mid-catagen-like, $n = 1 \times 3$), and 12 (late catagen-like, $n = 2 \times 3$) days in culture, respectively. Each extraction contained three hair follicles, was measured in duplicate, and produced highly reproductive results. Amount of mRNA is presented in arbitrary units compared to control levels, when control levels equal 1. **C:** Nine human hair follicles were cultured with or without 50 ng/ml of 7S NGF throughout 48 hours. Proximal (lower) portions were collected with surgical scalpels after the culture. The expression of TGF-β2 was measured by quantitative real-time PCR (TaqMan PCR) and normalized with GAPDH expression. Each PCR reaction was performed in triplicate. Graphs represent pooled date from these triplicates. Significance refers to control levels (*P* values by Mann-Whitney *U*-test: $<0.01 = ++$). **D:** Hair follicles were cultured in the presence of 5 or 50 ng/ml pro-NGF/NGF and TGF-β-neutralizing antibodies. Data pooled from two independent donors (nine hair follicles per experiment) are shown. Growth rates are expressed as percent difference over day 0. A significant difference in hair shaft elongation was detected between control hair follicles and hair follicles treated with 50 ng/ml of pro-NGF/NGF and TGF-β2-neutralizing antibodies after 4 days in culture (*P* values by Mann-Whitney *U*-test: $<0.05 = +$). ch, club hair; dp, dermal papilla; es, epithelial strand; hm, hair matrix; irs, inner root sheath; ors, outer root sheath.

levels during spontaneous catagen-like development of cultured human hair follicles (Figure 8B).

Human scalp skin anagen hair follicles that were cultured in the presence of 7S NGF were harvested after a

culture period of 4 days to determine whether pro-NGF/NGF treatment resulted in up-regulation of the catagen promoter TGF-β2,[31,32,42,43] at a time point when histomorphometry (not shown) and Ki67/TUNEL-analysis (Figure 6) did not yet indicate a catagen-promoting effect of pro-NGF/NGF in cultured human anagen scalp skin hair follicles. By quantitative PCR analysis of TGF-β2 steady-state mRNA levels in hair bulbs, we found a substantial up-regulation of TGF-β2 mRNA in hair follicles cultured in the presence of 50 ng/ml of 7S NGF as early as 2 days after onset of culture (Figure 8C). When hair follicles were cultured in the presence of pro-NGF/NGF and TGF-β-neutralizing antibodies, the catagen-promoting effect of 50 ng/ml of pro-NGF/NGF was neutralized, and hair growth was significantly promoted in hair follicles treated with 5 and 50 ng/ml of pro-NGF/NGF and TGF-neutralizing antibodies after 4 days in culture (Figure 8D).

Discussion

We here report that the human hair follicle is both a source and a target of pro-NGF/NGF signaling, suggesting a role for pro-NGF/NGF during anagen-catagen transition. First, pro-NGF and NGF are able to signal within the human hair follicle through their present receptors (Figures 1 and 2). Second, the expression patterns of pro-NGF, NGF, TrkA, and p75NTR in human anagen VI scalp hair follicles are distinct and change dramatically during anagen-catagen transition (Figures 2 and 3). Third, the expression pattern of p75NTR corresponds to the intrafollicular foci of keratinocyte apoptosis during catagen development in culture (Figures 3 and 4). Fourth, catagen development in organ-cultured human scalp skin anagen hair follicles is promoted by pro-NGF/NGF and inhibited by p75NTR-blocking antibodies (Figures 5 to 7). Finally, pro-NGF/NGF-induced hair follicle regression involves up-regulation of and signaling through the catagen-marker TGF-β2 in the human anagen hair follicle (Figure 8). Therefore, we conclude that the human scalp skin hair follicle holds the full capacity for pro-NGF and NGF signaling via TrkA and p75NTR and that pro-apoptotic signaling is facilitated in the catagen hair bulb. Subsequently, we discuss in more detail the pathways traveled by intrafollicular pro-NGF/NGF-signaling.

We were able to delineate an apopto-map of pro-NGF, p75NTR/TUNEL, and TGF-β2 expression in the regressing human hair bulb, corresponding to what we had previously characterized for the regressing murine hair follicle.[27] This apopto-map suggests that p75NTR is a key receptor on hair follicle keratinocytes in the regressing epithelial compartments (Figures 3 and 4). Accordingly, we found that pro-NGF/NGF treatment induces apoptosis and TGF-β2 expression in organ-cultured human anagen hair follicles dependent on the applied pro-NGF/NGF concentration (Figures 5 to 8). The p75NTR activation in human neurotrophin-induced hair follicle regression thus resembles the catagen induction pathways by neurotrophins reported earlier in mice.[18,23,26]

Expression of pro-NGF in human skin had heretofore only been described once, and this study described expression in human basal epidermal keratinocytes and in human cutaneous nerve fibers.[44] similar to the expression patterns we describe here, pro-NGF has also been demonstrated in ovine anagen hair follicles.[45] Both studies, however, have not co-analyzed the respective receptors and provide no corresponding functional experiments.

As indirect support of our hypothesis that p75NTR plays a major role in human neurotrophin-induced hair follicle regression, hair follicles derived from donors with low hair shaft elongation in control hair follicles (~60%) improved after p75NTR-neutralization (Figure 8). These hair follicle cultures were characterized by early entry of control hair follicles into a catagen-like development resulting in early cessation of hair growth, always accompanied by high expression of p75NTR in the regression hair bulb (not shown, compare Figure 3). Thus, these experiments can be compared to murine organ culture experiments with late anagen-skin before entry into catagen, in which p75NTR-expression predominates and pro-NGF/NGF induces catagen. In our human hair follicles from ill-growing donors, p75NTR predominates early on, and hair follicles profit from its blockade. Also, we had previously shown that another neurotrophin, brain-derived neurotropic factor (BDNF), which can signal through p75NTR but not through TrkA, also up-regulates TGF-β2 and induces premature catagen entry in organ-cultured human hair follicles.[31] Unfortunately, we were unable to conduct our experiments with a specific p75NTR-antagonist, as we had done in mice, since this specific agonist has not yet become commercially available (the same is true for selective TrkA ligands).[23,46]

In contrast, hair follicles from donors showing high hair shaft elongation (~100%) showed reduced hair shaft elongation under p75NTR-blocking conditions (Figure 8). These hair follicle cultures were characterized by late entry of control hair follicles into a catagen-like stage, low p75NTR-expression in the hair bulb and p75NTR co-expression with TrkA in the outer root sheath until late in culture (not shown). Possibly, these experiments can be compared to murine organ culture experiments with early anagen skin, where TrkA/p75NTR co-expression predominates and NGF promotes hair growth.[31] In our human hair follicles, p75NTR neutralization thus interfered with endogenous NGF/TrkA/p75NTR signaling and resulted in premature regression.

On the mRNA level, we found that pro-NGF/NGF was able to induce increased TGF-β2 message expression as early as 2 days after onset of culture (Figure 7). TGF-β2 up-regulation leads to activation of caspases 3 and 9 in the distal hair follicle epithelium and thereby to apoptosis of epithelial cells.[32,43] This up-regulation is especially interesting in light of our finding that hair follicles, left to spontaneously enter a catagen-like development in culture, show dramatically up-regulated TGF-β2-levels after 4 and 8 days in culture, before the up-regulation of p75NTR. Thus, before exerting direct pro-apoptotic effects on the regressing hair bulb through p75NTR signaling, pro-NGF/NGF may act as a catagen promoter by up-regulating TGF-β2. This is further supported by the fact that cultured hair follicles with increased TGF-β2

expression in the regressing hair bulb (compare Figure 3) display anagen to early/mid-catagen-like morphology (not shown), which is characterized by increasing apoptosis in the regressing hair bulb. In contrast, apoptosis is most prominent during the final stages of spontaneous catagen-like hair follicle involution in culture, when p75NTR mRNA as well as p75NTR protein expression in the hair bulb are maximal (Figures 3 and 4). Equipotent apoptosis induction by TGF-β and NGF had been shown in the retina, and the lack of an additive effect in this context had been interpreted as acting through the same channel.[47] Our observation of TGF-β2 involvement in BDNF-induced hair follicle regression further supports this notion.[31]

Taken together, these findings indicate that on the one hand pro-NGF/NGF induces hair bulb keratinocyte apoptosis and promotes catagen via p75NTR-dependent pathways, which includes the up-regulation of TGF-β2 as a key catagen promoter.[42] On the other hand, pro-NGF/NGF may be able to promote survival and/or stimulate proliferation of selected hair follicle keratinocytes populations via TrkA/p75NTR-dependent pathways—as has been reported, for example, in the epidermis of psoriatic skin[48] and in healing wounds.[10,49]

Thus, our data support the concept that NGF operates like a double-edged sword in cutaneous biology and promotes proliferation in some cell populations yet promotes apoptosis in others, depending on their neurotrophin receptor (co-) expression pattern.[7,8,16] To calculate and predict the net effect to be expected by pro-NGF/NGF application, for example in the treatment of hair growth disorders, it may therefore be necessary to determine receptor expression before treatment. The same should apply to other neurotrophin-controlled tissues characterized by differential receptor and ligand expression, such as the healing epidermis or peripheral neuropathies.

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